Review article

The horizon of bone organoid: A perspective on construction and application

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ABSTRACT

Bone defects repair and regeneration by various causes such as tumor resection, trauma, degeneration, etc. have always been a key issue in the clinics. As one of the few organs that can regenerate after adulthood, bone itself has a strong regenerative ability. In recent decades, bone tissue engineering technology provides various types of functional scaffold materials and seed cells for bone regeneration and repair, which significantly accelerates the speed and quality of bone regeneration, and many clinical problems are gradually solved. However, the bone metabolism mechanism is complicated, the research duration is long and difficult, which significantly restricts the progress of bone regeneration and repair research. Organoids as a new concept, which is built in vitro with the help of tissue engineering technology based on biological theory, can simulate the complex biological functions of organs in vivo. Once proposed, it shows broad application prospects in the research of organ development, drug screening, mechanism study, and so on. As a complex and special organ, bone organoid construction itself is quite challenging. This review will introduce the characteristics of bone microenvironment, the concept of organoids, focus on the research progress of bone organoids, and propose the strategies for bone organoid construction, study direction, and application prospects.

1. Introduction

Bone disorders have been the huge social burden that affect tens of millions of people around the world. Bone and joint diseases, such as osteoporosis, osteoarthritis, and bone-related cancers are financially daunting because of long-term curing periods [1]. It is increasingly required more insight into the biomechanisms, cues, developments, and therapeutics of bone-related diseases [2]. Currently, preclinical models for bone research mainly depend on the 2D cell culture of mammalian cells and animal experiments. The former is composited by one type of primary or immortalized cell line in a form of a monolayer cell sheet. Cells on the flat dish via conventional 2D culture are featured with 2D simplistic interactions and are homogeneous when exposed to drugs or other molecules. However, it is far away from the natural physiological microenvironments, leading to wrong results and causing huge financial costs. The simplistic nature of 2D culture makes it difficult to recapitulate the comprehensive microenvironments in the natural bone niche [3]. To overcome the gap, preclinical animal models are applied to imitate the in vivo environment. However, they are limited by the physiological differences between nonhuman species and humans, as well as the high costs. Therefore, the 3D systems in vitro that recapitulate physiological relevance have become a promising alternative for bridging the gap between 2D monolayer cell culture and living animals in vivo [4].

Bone organoids are 3D self-renewing and self-organized micro-bone tissues with biomimetic spatial features which are built based on bioactive materials and directionally differentiated from stem cells (such as bone stem cells, embryonic stem cells, etc.) or progenitor cells (such as osteoblast and/or osteoclast, etc) [5]. To realize the construction of biomimetic structures, a series of biocompatible materials, including Matrigel and synthesized alternative hydrogels, are applied to support the self-organization of bone organoids. As a typical example of 3D cell culture, bone organoids allow presenting the physiological oxygen and metabolic gradients, also maintain the extracellular and intercellular junctions [6]. Distinguished from other 3D cell culture models (such as spheroids), bone organoids are originated from human-specific tissue

and self-organize into organ-like tissues. While spheroids are close-packing cell aggregates with the random distributions. Even though the common bone tissue engineering would introduce the 3D cellular interactions, they are still failing to represent the real physiological microenvironment due to unfit cell types and too much human manipulation. From these views, bone organoids seem to be the ideal models that reflect the complex organ-like physiological microenvironment for the personalized medicine research [7]. Bone organoids can be applied for the research of bone formation, regulatory mechanism, tissue regeneration and open new access to gain insight into the mechanism, diagnosis, and thereby precaution of diseases.

Recently, biologically active materials have attracted great attention for their capacity of modulating the fate of stem cells [8]. Natural tissues are well-organized and hierarchical with various spatial and temporal characteristics [9]. In the natural physiological microenvironment, stem cells are exposed to numerous cytokines and growth factors, accompanying with comprehensive crosstalk with surrounding cells. In the process of organoid formation, it is difficult to recapitulate all the cues in vitro. Therefore, active biomaterials are designed to support the generation of close to real organoids. Various material factors such as porosity [10], polymeric chemistry [11], stiffness, and charges would be flexibly modulated to affect the differentiation of stem cells, so-called materiobiology [12].

It is well known that bone is a living organ that contains a bulk of bone cells and immune cells in its bone niche. Bone is the mechanical support for the human body, which is bearing mechanical loading all the time during the whole life [13]. Bone is dynamically remodeled by the balance between bone formation and bone resorption, which mainly are executed by osteoblasts and osteoclasts. The bone organ is also composited by hard scaffolds that are biomineralized by hydroxyapatite and collagen [14]. In principle, the construction of bone organoid would be intriguing and challenging. To this end, it is of vital significance to explore the composition and cellular interaction within bone microenvironment in the first section. Then, the main concerns for the construction of bone organoids were discussed in the following section.

Development of bone organoids is urgently required to represent the close to natural cell features, such as activated molecular pathways, biological outcomes, and phenotype. Finally, bone organoids are promising as a powerful tool for modeling diseases and testing systems for drug development.

2. Bone microenvironment: basis for bone organoids

The bone microenvironment is the theoretic basis of bone organoids, which aim to simulate the real bone marrow. The bone microenvironment is a unique, highly dynamic compartment composed of heterogeneous cells, extracellular matrix, soluble growth factors, and cytokines [15] (Fig. 1). Functionally, bone microenvironment contains mainly three units, including bone formation unit, bone resorption unit, and hemopoietic unit [16,17]. Bone marrow mesenchymal stem cells (MSCs) are a group of cells with multidirectional differentiation potential, responsible for the differentiation into non-blood cell components, mainly including osteoblasts, adipocytes, chondrocytes, and fibroblasts. MSCs and differentiated osteoblasts form bone formation unit and osteoclasts are mainly responsible for bone resorption. Bone marrow hematopoietic stem cells is the source of blood cells and immune cells in the systemic circulatory system [18].

3. Functions and signaling pathways in bone organoids design

Learning how each functional units build, and work is the prerequisite for bone organoids construction. Through temporal and spatial induction in vitro with certain cytokines activating signaling pathways, we could control the direction of organoids building.

3.1. Bone formation unit

Bone formation is one major bone function associated with most bone disorders like osteoporosis, osteoarthritis et al. To simulate bone formation function in vitro is one major target of constructing bone
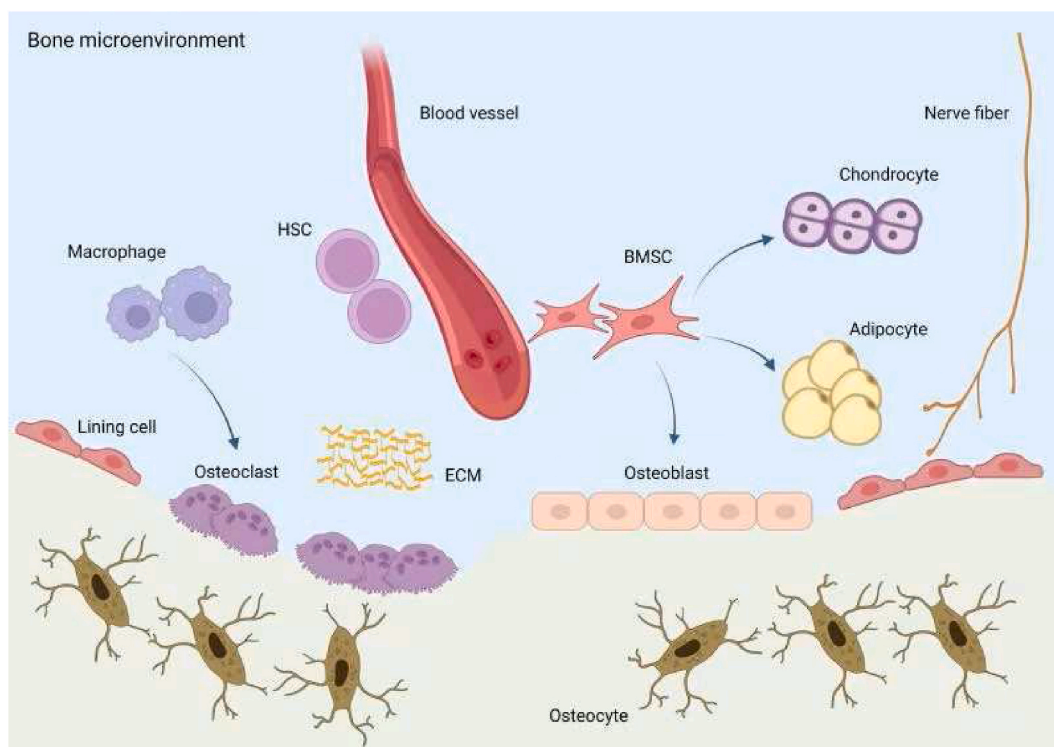

Fig. 1. Bone microenvironment. Bone marrow mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC) and their descendants forms three functional units: bone formation unit, bone resorption unit, and hemopoietic unit.

organoids. MSCs are the source of bone-forming cells. According to the International Society of Cell Therapy's definition, MSCs are described as a heterogeneous group of cells that are able to adhere to plastic and can be isolated by a range of phenotypes; in addition, it can differentiate into adipose, bone, and cartilage [19].

The osteo-lineage cells include osteoprogenitors, preosteoblasts and osteoblasts [20]. Osteoblasts are the main cells involved in bone formation. Extracellular matrix proteins can be secreted by these cells to regulate the process that calcium is mineralized in the form of hydroxyapatite and type I collagen, which provides structural support for the skeleton.

The bone forming unit is composed of a specific set of components in the cellular microenvironment (Fig. 2A). Despite the wide variety of molecules involved, Runt-related transcription factor 2 (RUNX2) and Osterix are two critical translation factors (TFs) that promote osteogenic differentiation of MSCs [21]. RUNX2, activated by AP-1, CBFβ, CBP, P300, Smad1/5, can promote osteoblast differentiation by targeting key components of the pathway, such as BMP2 and Smad1 [22–24]. Many other factors, including bone morphogenetic protein-2 (BMP-2), Dlx5, Sprouty 2 (Spry2), Twist-1, and Twist-2, can upregulate the level of Runx2 and then coordinate with it [25–28]. Under pathological conditions, unintended upregulation of Runx2 will result in ectopic ossification [29]. By targeting Runx2/Cbfa1, BMP2 plays a key role in signal transduction. With the increase of concentrations, BMP2 shows a dose-dependent effect on osteogenesis [30]. Besides, osterix is an additional important TF for osteogenesis. Osterix is activated by RUNX2 and play a role downstream of RUNX2 [31]. In osterix-null mice, MSCs fail to differentiate into osteoblasts, and no osteogenesis occurs [31].

The other major fate of MSCs differentiation is adipocyte. The balance between osteoblasts and adipocytes is vital for bone formation as well as bone resorption. The adipo-lineage cells include adipoprogenitor, preadipocyte and adipocyte [32]. Adipocytes are one of the most common cell types in the adult skeleton; both mature adipocytes and preadipocytes act as endocrine cells that secrete a number of soluble molecules into the microenvironment [32].

PPARγ plays an important role in adipogenesis by regulating the expression of adipogenic genes. It shows adipopogenesis-promoting and osteogenesis-inhibiting effects [33]. PPARγ is a secretory BMP inhibitor [34]. As mentioned above, high concentrations of BMP2 accelerated osteoblastogenesis, whereas low concentrations of BMP2 promoted differentiation of the C3H10T1/2 mesenchymal cell line to adipocytes [35]. In addition, CCAAT/enhancer binding protein α (C/EBPα), platelet growth factor receptor β and zinc finger proteins 423 and 521 also promote lipogenic differentiation of MSCs [36–39].

3.2. Bone resorption unit

Bone resorption, as coupled physiological processes with bone formation, can precisely regulate bone homeostasis. Originating from the monocyte/macrophage lineage, osteoclasts become mature after fusion of mononuclear precursor cells. Highly expressed tartrate resistant acid phosphatase and cathepsin K are the main markers of osteoclasts [40]. Then, osteoclasts can immigrate into bone via the bloodstream and resorb mineralized tissues [41–43].

The differentiation process of osteoclasts is regulated by a series of cytokines (CK) (Fig. 2B). Osteoclasts are formed by the fusion of
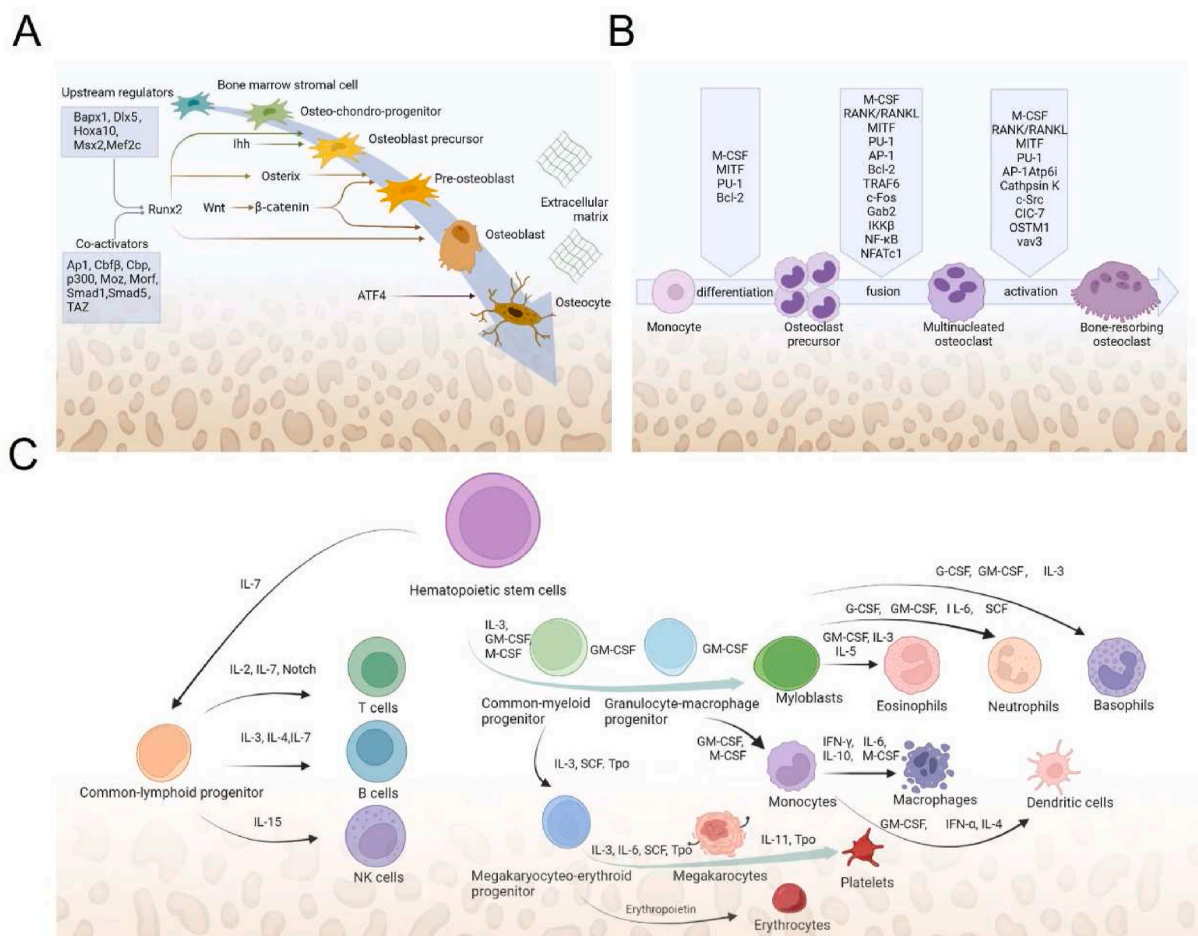

Fig. 2. Functional unit. (A) Bone formation unit: the differentiation route of MSC into osteocyte; (B) Bone resorption unit: the formation of mature osteoclast; (C) Hemopoietic unit: A spectrum of human HSC differentiation.

mononuclear progenitors of the monocyte/macrophage family. Macrophage colony-stimulating factor 1 (M-CSF) and receptor activator for nuclear factor-κB Ligand (RANKL) are two key CKs that promote osteoclast differentiation [44]. M-CSF is a stable dimer expressed by bone marrow monocytes. It regulates the differentiation of macrophages into osteoclast precursors in the early stage and mainly promotes the expression of NF-κB receptor activator in osteoclast precursor cells. M-CSF can regulate the proliferation and apoptosis of osteoblasts and their precursor cells by combining with osteoclast precursor cells [45]. In addition to M-CSF, RANKL is another primary CK for the osteoclastogenesis. RANKL from stromal/osteoblasts binds RANK on osteoclast precursors, thus promoting signaling by recruiting adaptor molecules after ligand-induced trimerization [46], targeting key factors of the pathway, such as NFATc1 and TRAF6, PU.1 MITF, AP-1 [47], thus inducing the formation of mature osteoclast that resorb bones.

3.3. Hemopoietic unit

Hematopoietic stem cells (HSC) are adult stem cells in the blood system, a heterogeneous population with the ability to self-renew, proliferate and differentiate into mature blood cells of all lineages [48] (Fig. 2C). Hematopoietic stem cells can be obtained from umbilical cord blood, bone marrow and adult peripheral blood. Most hematopoietic stem cells are in a resting state and are activated upon external stimulation [49].

3.3.1. Lymphoid cells
Upon IL-7 stimulation, HSC differentiates into common lymphoid progenitor cells [50]. In response to stimulation by IL-3, IL-4, and IL-7 cytokines, lymphoid progenitor cells differentiate into B lymphocytes, which have differentiated and developed from lymphoid stem cells in mammalian bone marrow or avian bursa. Mature B cells are activated and turned into plasma cells after antigenic stimulation by microorganisms and plasma cells produce antibodies to participate in the humoral immune process [51].

Lymphatic progenitor cells are stimulated by IL-15 to differentiate into natural killer cells (NK cells) [52]. Human NK cells show TCR^-, mIg^-, $CD56^+$, and $CD16^+$ phenotypes. Activated NK cells can exert immunomodulatory effects through cytokines such as IFN-γ and TNF-A [53].

Lymphatic progenitor cells are stimulated by IL-2, IL-7 and Notch to differentiate into T cells [54]. Mature T cells are found mainly in the peripheral thymus and are involved in not only cellular but also humoral immune processes. In addition, lymphoid progenitor cells differentiate into dendritic cells in response to stimulation by Flt-3 Ligand [55].

3.3.2. Myeloid cells
HSC differentiate into common myeloid progenitor cells under IL-3, GM-CSF, and M-CSF stimulation [56]. Myeloid progenitor cells can differentiate into granulocyte-macrophage progenitor cells and megakaryocyte-erythroid progenitor cells under different stimuli. Megakaryocyte-erythroid progenitor cells are stimulated by erythropoietin to produce erythrocytes and can be stimulated by IL-3, IL-3, SCF, and Tpo to produce megakaryocytes. Megakaryocyte is huge in size, and mature megakaryocytes form platelets after partial rupture upon IL-11 and Tpo stimulation at the edges [57,58].

Granulocyte-macrophage progenitor cells can differentiate into primitive granulocytes and monocytes under different stimuli. Monocytes are generated with the stimulation of GM-CSF, M-CSF, and monocytes differentiate into monocyte-derived dendritic cells in response to Flt-3 Ligand, GM-CSF, IFN-alpha and IL-4 stimulation [59]. Monocytes differentiated into macrophages stimulated by IL-6, IL-10, M-CSF and IFN gamma [60].

Myeloblasts are generated by GM-CSF, and are stimulated by G-CSF, GM-CSF, IL-6, and SCF to generate neutrophils. Neutrophils are chemotactic, phagocytic and bactericidal. Primitive granulocytes are stimulated by GM-CSF, IL-3 and IL-5 to produce eosinophils. Myeloblasts are stimulated by GM-CSF, IL-3, and G-CSF to generate basophils [61, 62].

4. Bio-technology of bone organoid

The development of bone organoids is still in its infancy, due to the few understanding of biomechanisms of bone-related diseases and the difficulty in directional differentiation stem cells. There are several key steps to establish a bone organoid (Fig 3): firstly, it is necessary to confirm the cell sources. The selection of stem cells would decide the physiological pathways to educate the cells with targeting cellular functions. Then, the biomaterials were introduced as the matrix for the growth and differentiation of 3D cellular organoids. Moreover, it needs to employ some constructing methods to build the 3D spherical organoids [14].

4.1. Cells for bone organoid

Accordingly, organoids start from stem cells, which can be edited to present the phenotype and mutation of interest. As mentioned above, bone formation, bone resorption, and hemopoiesis are the three main dynamic processes in bone niche. Correspondingly, the functions of bone organoids can be well controlled via using different type of bone-related cells, namely osteoblasts (which build bone tissue), osteoclasts (which take bone away) and osteocytes (which regulate the building and breaking down of bone) [63].

To construct a basic bone organoid for bone disease research, osteoblasts are primarily considered, for their critical roles in bone building. Autologous or allogeneic stem cells, such as human induced pluripotent stem cells (iPSCs), multipotent mesenchymal stem cells (MSCs) [64], human-periosteum-derived cells (hPDCs), and embryonic stem cell (mESC)-derived osteoblast/osteocyte population, exhibit great potentials for the fabrication of bone organoids.

Firstly, induced human pluripotent stem cells (iPSCs) are populations that are reprogramed from fibroblasts and have the potential for many different cell fates. Compared with other types of stem cells, iPSCs processes a huge advantage in cellular origins. It is more accessible as autogenous fibroblasts can be isolated in demand. Meanwhile, they can be reprogramed into a variety of cell types and construct complex systems with a consistent genotype. Importantly, iPSCs also allow for precise patient-specific manner. They keep the genotype from patient, which would greatly present the patient-specific models for bone disorders, and establish models for drug testing and drug discovery.

In the natural bone microenvironment, marrow stromal cells, most representative multipotent mesenchymal stromal cells (MSCs), can be recruited from bone marrow into bone organs and differentiate into osteoblasts [65]. Herein, directly reconstruction of this process in vitro might provide a convenient pathway to gain a bone organoid. More interesting, the stemness, proliferation and differentiation of MSCs were demonstrated to be gradually improved in a 3D culture conditions. It has been demonstrated that the anti-inflammatory and antiapoptotic performances of MSCs were also beneficially modulated. It has been demonstrated MSC derived spheroids confirm the presence of osteocytes and osteoblasts. In addition, bone organoids from MSC are probably immune compatible for a bone tissue regeneration.

Similar to MSCs, progenitor cells exhibit outstanding cellular characteristics for bone formation potency. The periosteum supplies the osteoblasts, nutrients, and blood vessels for bone metabolism. Upon bone damages, periosteum response to ROS stress and mechanical stress, then rapidly execute the generation of osteoblasts for bone formations [66]. It has been reported that the mechanical stretch or small molecules can be applied to activate the differentiation of progenitor cells. Herein, the bone organoids from periosteum cells would offer an efficient for rapid self-organization of bone models.

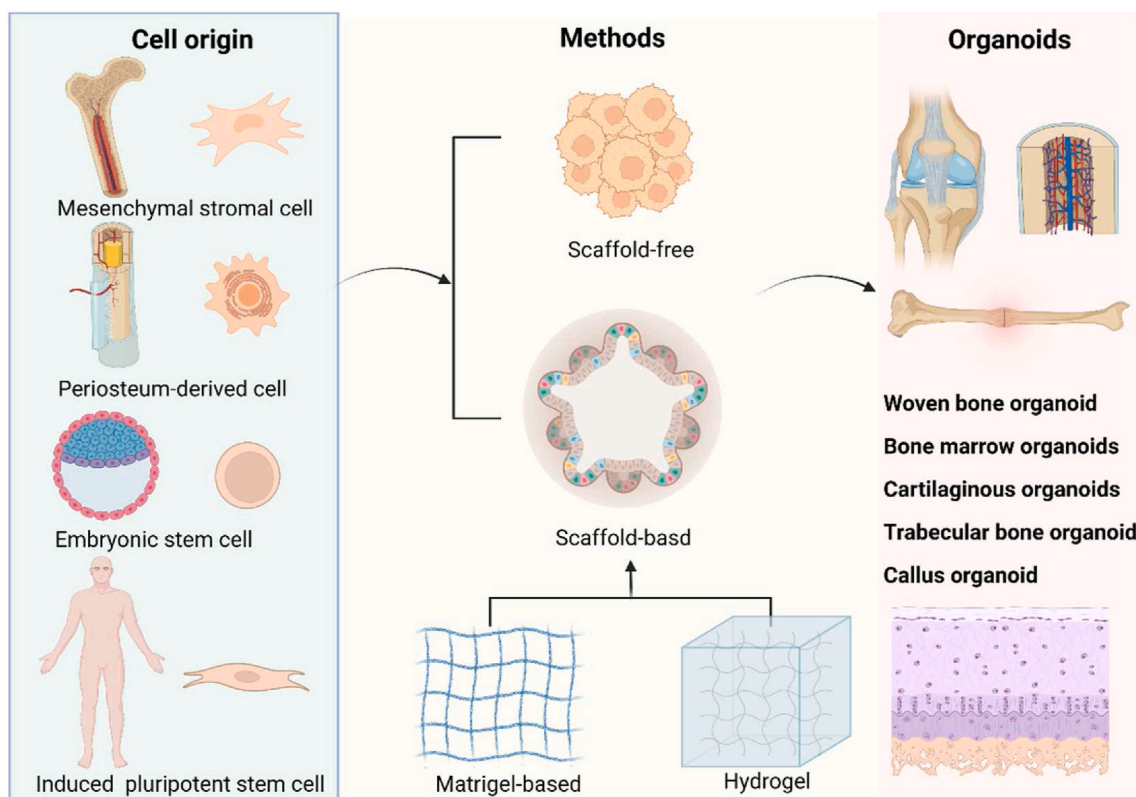More popularly, the embryonic stem cells are widely applied to

Fig. 3. A schematic figure of biofabrication technique for bone organoid. Firstly, the stem cells are isolated for differentiation. Then, cells aggregate into a scaffold-free or organoids. Then a series of diseases models can be represented.

produce a series of organs for the multipotency [67]. During the past decades, numerous organoids such as liver, gastrointestinal, pancreas have been widely engineered from embryonic stem cells. Embryonic stem cells are more versatile but are also controversial. This is because the isolation of these stem cells might destroy the embryo structure and disturb the normal growth of life.

In addition to the generation of bone formation unit, it would be intriguing to build the bone resorption unit. Osteoclast group is the main executor of bone resorption. These cells are derived from hematopoietic cells, such as macrophages. As mentioned above, the bone organs always exposed to mechanical loading. For the health bone organs, the bone resorption helps to gain the confine and compact structure. The bone formation and bone resorption are usually modulated by couple of signaling pathways, such as RANK-RANKL signaling. Essentially, the RANK receptors expressed on osteoclasts can be modulated by a soluble RANK ligand (RANK) that is produced by osteocytes [13]. The osteoblast-osteoclast crosstalk might provide a window for the developing mechanism of bone-related diseases. It would be an intriguing model to synchronously represent both the bone formation unit and bone resorption.

The final function of bone is hemopoiesis. The bone marrow (BM) is demonstrated to tightly modulate the organs and home to hemopoietic cells and progenitor, as well as bone marrow related stomal cells. These cells in bone marrow play critical role in connective tissue regeneration and vascularization. To construct BM organoids model in vitro, Ehrbar et al. employed the poly (ethylene glycol) (PEG) and hyaluronic acid (HA) to mimic the bone marrow niche. Then human bone MSC (hBMSC), hemopoietic cells and progenitor (hSPC), as well as bone marrow related stomal cells were incubated. These cells maintain stemness and initiate the differentiation upon the application of soluble factors including bone morphogenetic protein-2 (BMP-2), resulting in well-defined BM organoids.

4.2. Biomaterials for bone organoid

In general, the organogenesis in vivo exhibits distinguished spatio-temporal features which are synchronously the microenvironment and genetic programming, involving numerous cytokines and growth factors (Fig. 4). The cytokines and growth factors are usually released from various cells in the tissue niche. However, the organoid formation in vitro is commonly dependent on the spontaneous self-organization and self-renewing of stem cells. These dynamic processes might undergo cell proliferation, migration, differentiation and 3D organoids formation and growth. Compared with in vivo process, it would be more difficult to control the morphologies of organoids. Herein, various biomaterials were designed to recapitulate the natural extracellular matrix conditions for specifying the stem cell fate [10].

In principle, the construction of bone organoids in vitro mimics the organogenesis of bone development in vivo, ending up with bone-specific functions. In living body, the organogenesis involves abundant ECM generation and dynamic organization of ECM network. The ECM not only supports the adhesion, growth, spreading, and differentiation for cells, but also acts as a critical role in the spatiotemporal control for organs [11]. Herein, the application of various ECM-derived materials has been demonstrated as an efficient approach to accelerate the construction of bone organoids. Intrinsically, ECM is the mixture of wide range of proteins, including fibrillar proteins (e.g., collagens, elastin), glycoproteins (e.g., fibronectin, laminins), proteoglycans (e.g., perlecan, syndecan), glycosaminoglycans (e.g., heparan sulfate (HS), hyaluronan (HA)). A great number of ECM derived biomaterials have been selected, because of the biocompatibility and the close to real chemistry.

As a typical ECM derived material, Matrigel and tissue-specific extracellular matrix have been shown to support efficient organoid development [68]. The Matrigel is a natural ECM that is produced by mouse osteosarcoma. Contributing to the ECM chemistry, Matrigel provides abundant collagen supports and biocompatible physiological
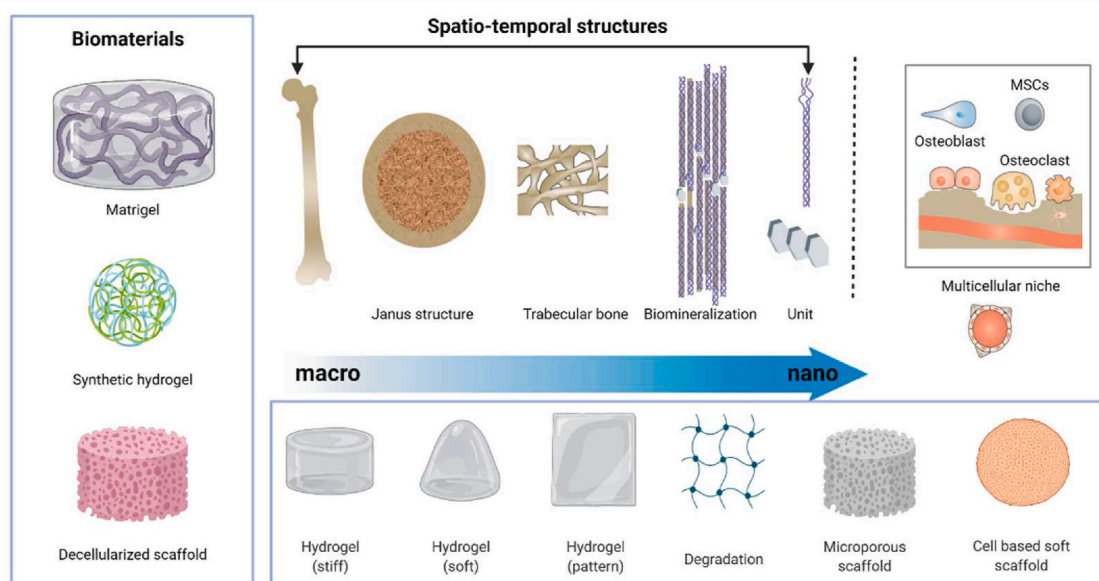
Fig. 4. The biomaterials interact with stem cells. The bone tissue is featured with hierarchically spatiotemporal characters spanning from macro-to-nano scales. To generate a bone structure in vitro, the biomaterials with various features including the stiffness, the micropatterns and structures are developed.

feature for growth of organoids. Meanwhile, there are numerous growth factors are loaded in Matrigel to maintain the stemness or trigger the differentiation of stem cells. Due to the outstanding performances in supporting cellular growth, Matrigel has been adopted to tissue development processes, disease modeling, and drug screening. However, Matrigel is high cost, and present chemically undefined nature. What's more, the batch-to-batch variations lead to a worse reproducibility. Notably, the natural ECM derived hydrogels are inherent biological activity and become degradable during the formation of bone organoids, ending up with too weak mechanics to support the full organogenesis and often presenting underdeveloped organoids.

To address these, great efforts have been devoted to designing the synthetically ECM-mimicking materials. Both natural and synthetical biomaterials have been applied to improve the experimental reproducibility. Thereby, collagen, gelatin, alginate, fibrin, and hyaluronic acid (HA) have been applied to incubate the organoids [69–73]. Polyethylene glycol (PEG) and polyisocyanopeptide (PIC) are the two pioneer synthetic polymers used closely in organoid research. Among them, PEG-based hydrogels are most widely applied for the flexibility in chemical modification, molecular weight modulation, crosslinking, allowing for a wide type of organoids fabrications.

In vivo, Bone is a complex system of cavities and channels, called the lacuna-canalicular-network (LCN). The bone cell behaviors and functions can be affected by the mechanical properties of matrix microenvironments. Mechanical performances of the hydrogel matrix can decide the cellular behaviors including attachment, spreading, migration and differentiation. Cell sense the mechanical strength from the adhered substrate via a Yes-associated protein (YAP) pathway and Hippo pathway [74]. As a result, cells can sense the topological geometries and stiffness from the ECM and biomaterials. Moreover, YAP plays a dual direction signals conduction between the nucleus and cytoskeleton. In this aspect, the mechanical pathways offer significant window to get insights into the mechanisms between the mechanics of biomaterials and cells or tissues. For example, mesenchymal stem cells on stiff substrates (>30 kPa) tend to generate more stress fibers and focal adhesions and prefer osteogenic differentiation. While on soft substrates (<10 kPa), cell adhesion is highly suppressed, and cells undergo adipogenic differentiation [67].

4.3. Fabrication for bone organoids

Due to the rapid development of this field, the 3D cell culture of organoids can be divided into scaffold-free and scaffold-based types. The principle of cellular spheroidal formation is to prevent cells from spreading on the substrates and facilitate their self-aggregation. To this end, several strategies based on antifouling substrates, centrifugal force, superamphobic surfaces, forced-floating, centrifugal force, shear force, surface tension, and gravity have been developed. Moreover, the stirring bioreactors and rotating bioreactors (such as NASA bioreactors) have been applied to scale up prepare the scaffold-free organoids. However, due to the relatively large size distribution and the biocompatibility, it becomes hard to provide reproducible results. Hanging drop method is turned out to be a convenient method to prepare the organoids in a short time. By upside-down droplets of the cell suspension, the spheroids can be obtained as fast as 1 day. For this method, the volume of hanging droplets is also controlled below 20 μL to prevent droplets from falling away. It needs to mentioned out it is quite difficult to fresh the culture medium. Herein, the drop-hanging method is not available for long-term culture. To deal with this problem, the ultra-low adhesive (ULA) hydrogels based on alginate, collagen, and hyaluronic acid were applied. Due to the formation of hydration layer, the protein adsorption and cell adhesion can be fully blocked.

As mentioned above, Natural tissues are hierarchical with spatial and temporal characteristics. For bone organoid, the bio-scaffolds are always required to construct the hierarchical tissue structure. Meanwhile, the bone tissue is composed by a bulk of inorganic structures, so called biomineralization. To this end, a series of biomaterials have been adopted to assist the formation of hierarchical bone tissues [75].

Woven bone organoid: recently, an organoid for woven bone was developed by Hofmann [76]. They directionally induced the human bone marrow stromal cells to differentiate into a functional 3D self-renewing coculture of osteoblasts and osteocytes. In this model, the osteocytes were embedded under the collagen matrix. More importantly, the constructed system exhibited a living model that can produce the woven bone, namely osteogenesis in vitro.

Bone marrow organoids: as mentioned above, the bone marrow microenvironment is composited by a bulk of cells and maintains the capacity of hematopoietic functions [73]. The ability to produce trabecular bone with architectural and compositional properties similar

to those of natural bone, which offers a way to produce bones of pre-defined size and shape. It could represent a new method for the study of bone biology, remodeling and pathophysiology in vitro.

Callus organoids: cell-based products (2D cell sheets or 3D cell aggregates) exhibit promising potential in regenerative medicine. Recently, the scaffold-free spheroids derived from human periosteum (hPDCs) are used for the construction of callus organoids for the bong bone healing. In this strategy, the hPDCs were incubated into the 3D cell aggregates. The differentiation of stem cells was well regulated via a series of growth factors and inhibitors.

Cartilaginous: similar to callus organoids, the cartilaginous organoids can be self-assembled from human pluripotent stem cells into cartilaginous organoids. The scaffold-free cartilaginous organoids can be applied for the regenerative repair of critical size long bone defects.

Trabecular bone organoids: a trabecular bone organoid has been modeled via directing the osteoblasts to form mineralized bone tissue and acquire the bone lining cell phenotype. The primary murine osteoblasts and bone marrow mononuclear cells were applied to coculture the osteoblast and osteoclast. The trabecular bone organoids can be applied for the study of complex and dynamic regulation of bone remodeling process. Furthermore, another trabecular bone organoid was constructed from primary female osteoblastic and osteoclastic cells. The dynamic bone remodel was submitted to microgravity. It offers a new window for the study of the pathological bone loss and imbalances in bone remodeling.

5. Applications

5.1. Construction of bone disease model

The most significant advantage of organoid is humanized and physiological. Compared with traditional animal bone disease models, constructing human bone organoid in vitro can better simulate the pathological environment of human body, so as to clarify the mechanism of disease more accurately (Fig 5).

Osteoporosis model. Osteoporosis is one of the most common and popular bone diseases. Its performance is that the rate of bone resorption is greater than the rate of bone growth, which eventually leads to the decline of bone mineral density [77]. At present, osteoporosis models are generally established in animals by surgery, drugs, gene knockout and other methods, which not only have a long cycle but also have a high cost [78]. Constructing a bone osteoporotic organoid will greatly shorten the cycle and save the cost. For example, we can add RANKL/MCSF factors to mimic the microenvironment of osteoporosis and thus enhance the proportion and activity of osteoclasts. Compared with normal bone organoid, osteoporotic organoid should exhibit low mineral deposition (micro-CT analysis) and high trap activity (trap staining analysis). This can provide a new strategy for the construction of osteoporosis model.

Bone defect model. Bone defect is a bone shortage caused by trauma or surgery. The existence of bone defects often leads to bone nonunion, delayed healing or nonunion, and local dysfunction [79]. Bone defect models are commonly constructed by artificial destruction of animal cranium or leg bones [80]. By constructing bone organoid in vitro and then destroying it locally, we can simulate the real bone defect environment without sacrificing animals.

Bone tumor model. Bone tumor refers to the primary or secondary tumor of the human skeletal system and its related accessory tissues [81]. At present, the construction methods of bone tumor model mainly include cell suspension implantation and tissue mass implantation. However, implanted tumor cells or tissues are at risk of leakage and then affect other tissues [82]. By constructing bone organoid in vitro and inoculating tumor cells, the bone tumor organoid model can be constructed, which will eliminate the impact of leakage risk.

Bone malformation model. Bone malformation refers to the genetic defects of organs or parts of organs caused by abnormal fetal development [83]. Currently, Bone malformation models are mainly constructed by drugs or gene knockout, which have some disadvantages, such as long cycle, difficult modeling, large differences between groups and so on [84]. Through precise intervention and regulation, the
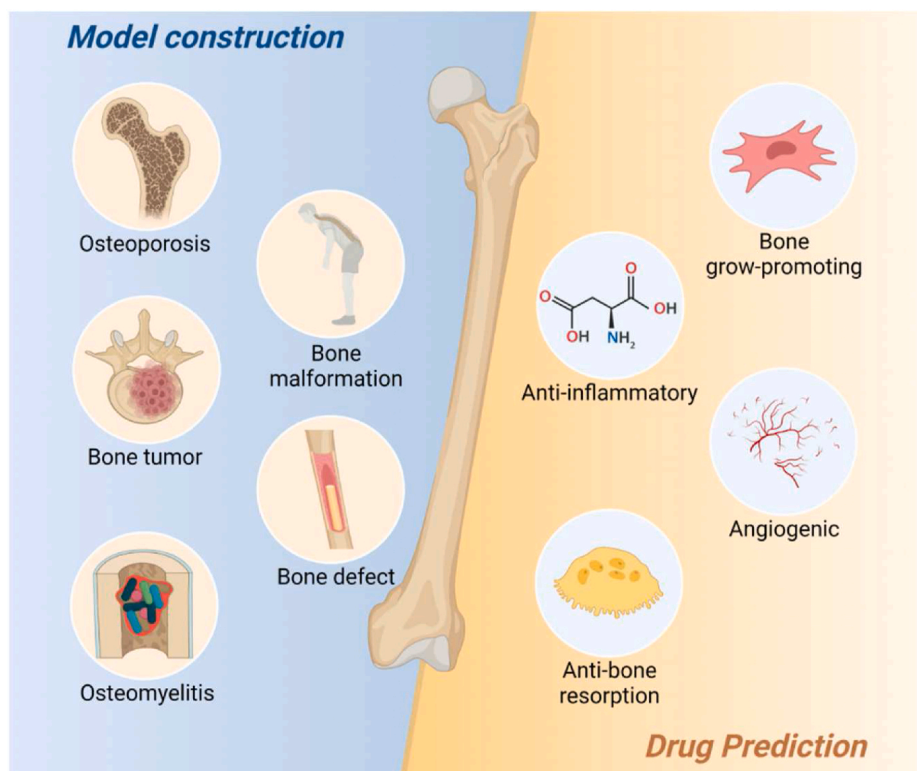

Fig. 5. The potential applications of bone organoid in construction of bone disease model and drug prediction.

construction of bone malformation organoid in vitro will solve the above shortcomings.

Osteomyelitis model. Osteomyelitis is a complex inflammatory bone disease, which is characterized by bone infection and bone destruction, mainly caused by bone microorganism infection [85]. At present, osteomyelitis models are generally established in animals by intravenous injection of *Staphylococcus aureus*, injection of *Staphylococcus aureus* into bone marrow cavity, or using implants loaded with *Staphylococcus aureus*. These methods are not only significantly different from the clinical pathogenic factors, but also easy to lead to the death of the model due to excessive bacteria [86]. By simulating the microenvironment of osteomyelitis, the construction of bone osteomyelitis organoid is not only more similar to the clinical pathogenic factors, but also will not give rise to the death of the model. This provides a novel strategy closer to the clinical practice for the construction of osteomyelitis model.

5.2. Drug prediction

The vast majority of clinical drugs for the treatment of bone diseases, such as anti-bone resorption drugs, angiogenic drugs, bone growth-promoting drugs, anti-inflammatory drugs, etc., need to undergo a long process of detection in vitro and in vivo before clinical approval [87]. These drugs are often terminated during phase I trials. It is noteworthy that long cycle and organ toxicity are two major disadvantages [88]. Currently, the conversion of commonly used cell and animal experimental results to clinical is not smooth. The construction of human bone organoid will greatly shorten the drug detection cycle and provide a more accurate mean for drug toxicity prediction [89]. Therefore, in drug research, especially for chronic bone diseases or the lack of large-scale clinical trials, bone organoid can provide sufficient resources for functional testing, phenotypic analysis and so on. Thus, bone organoid is an excellent model for drug toxicity prediction, new drug screening and individualized treatment.

5.3. Evaluation of implant biomaterials

Currently, the preclinical evaluation of bone implant biomaterials (such as medical metals, medical ceramics, medical polymers, etc.) is mainly at the expense of a large number of animals to obtain data [90]. Moreover, the data obtained by sacrificing these animals are often inconsistent in large-scale clinical trials due to species differences [91]. On the one hand, the construction of bone organoid can realize the in vitro detection of bone implant materials, so as to replace the in vivo evaluation and reduce animal sacrifice. On the other hand, the construction of human bone organoid can better simulate the human internal environment, avoid the data mismatch caused by species differences, and achieve an accurate preclinical evaluation of bone implant biomaterials (Fig 6).

5.4. Regenerative repair

The main goal of regenerative medicine is to replace a functional or damaged organ with healthy tissue in vitro, to achieve non-immunosuppression, disease-free and toxicity reduction, and to avoid the huge cost of lifelong anti-rejection therapy [92]. Modern medicine has been able to achieve autogenous bone/allogeneic bone transplantation, especially in the treatment of bone defects and bone tumors. Allogeneic bone transplantation is still the main clinical method, but there is a serious shortage of donors and tissue rejection [93]. Therefore, it is urgent to find new sources of bone tissue. Bone organoid can be amplified with homologous genetic tissue for auto transplantation and provide renewable resources for organ replacement strategies. For example, Gabriella et al. reported an engineered callus organoid [94]. The fabricated callus organoids could spontaneously bioassemble into large, engineered tissues and then heal murine critical-sized long bone defects. They found the regenerated bone exhibited similar morphology to native tibia. In addition, Tam et al. prepared a cartilaginous organoid and found that the cartilaginous organoid could promote scaffold-free healing of critical size long bone defects [66].

Apart from the above-mentioned applications, the bone-related cells and bone growth active factors produced by bone organoid can be used as bioactive components to induce the bone regeneration process. For example, Trubiani et al. found that stem cells cultured onto cortico-cancellous scaffold could enhance the osteogenic activity and
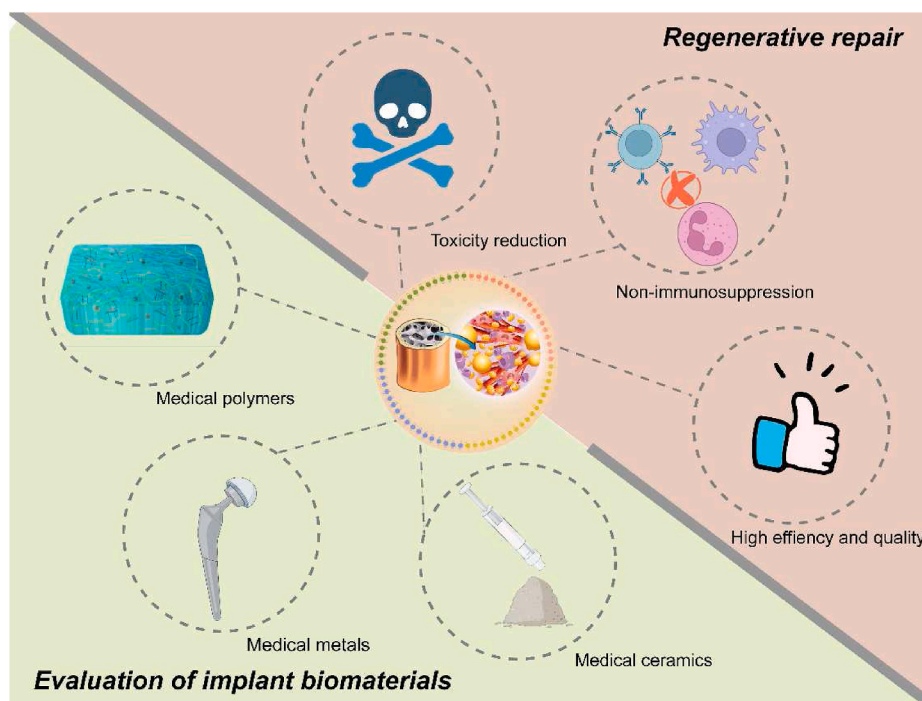

Fig. 6. The potential applications of bone organoid in evaluation of implant biomaterials and regenerative repair.

accelerate bone regeneration [95]. In addition, they also found that stem cells-derived extracellular vesicles contained huge amount of growth factors and could act as a big resource in regenerative medicine [96]. Such bioactive components produced by bone organoid are expected to have the ability to improve the regeneration and repair effect of traditional tissue engineering biomaterials.

6. Challenges and prospective

Even though the development of bone organoid is still in its infancy. The flexible design of biomaterials and directional differentiation of stemness cells are still burgeoningly investigated. There are promising potential applications for tissue engineering, which are still waiting for exploring. Even though, there are still some challenges that need to be addressed. Firstly, the reported bone organoids would represent only one functions for bone, such as bone formation, bone resorption or hemopoiesis. It is still a huge bottleneck to realize multi-function in an integrated bone organoid. Due to the complex cellular crosstalk, the direct coculture of different types of stem cells seems hard to control the direction of differentiation. The bottom-up strategy offers a possible approach to the hierarchical structures. Bone mini-organoids via scaffold-free methods can be treated as a building block for the 3D printing techniques, thus, promising in multifunctional bone tissue.

Another challenge is the 3D vascularization. As well known, the larger size tissue or even body contains abundant vascularization network to support the nutrient and oxygen supply. It is imageable that vascularization will be a prerequisite for large bone organoids. Inspired by the useful methods in bone tissue engineering, the vascular endothelial cells might be co-incubated to construct the vascularized organoids. Moreover, the growth factors, such VEGF, FGF are needed to stimulate the formation of vascular networks.

Upon the challenges, there are still some intriguing perspectives for this growing biological technique. Following recent advances in the understanding of organoid technique and microfluidic organ-on-a-chip, bone organoids would come to new age to burgeoning development. Meanwhile, the living systems are dynamic and bear mechanical stress, especially for bone tissue. By placing the bone organoids into a perfusion bioreactor or microfluidic chip, it is highly possible to recapitulate the biomechanics relationship among 3D organoid.

Then, with the advance of novel gene-editing methods, such as CRISPR/Cas9, it allows to precisely obtain a biomimetic tissue directly from the gene editing of healthy organoids. Given the versatility, gene editing tools help scientists to repeat the abundant bone disorders model for the development in drug and biomechanism research. Herein, the personalized or disease-specific bone organoids with candidate gene function would be used to investigate the some raw models in tissue physiology and carcinogenesis.

Finally but not the less, by integrating the emerging 3D printing skills or cell-based bottom-up fabrication, it is capable to realize the construction of biomimetic and hierarchical structures, including the bio-mineralization and spatiotemporally features. Overall, organoids have enormous potential to model development and disease, as a tool for drug testing, and as a therapeutic approach. Future efforts will no doubt bring them closer to reaching that potential.

Authorship contribution statement

Shuangshuang Chen, Zhen Geng, Xiao Chen contributed equally to this work. Han Liu, Yan Hu, and Xu Xue drafted Figures. These authors drafted and wrote the manuscript of this review. Jiacan Su guided and revised the manuscript of this review.

Declaration of competing interest

The authors declare no conflict of interest in this review.

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